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Development of a Complementing Cell Line and a System for Construction of Adenovirus Vectors with E1 and E2a Deleted

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Although adenovirus vectors offer many advantages, it would be desirable to develop vectors with improved expression and decreased toxicity. Toward this objective, an adenovirus vector system with deletion of both the E1 and E2a regions was developed. A 5.9-kb fragment of the adenovirus type 5 (Ad5) genome containing the E2a gene and its early and late promoters was transfected into 293 cells. A complementing cell line, designated 293-C2, expressed the E2a mRNA and protein and was found to complement the defect in Ad5 viruses with temperature-sensitive or deletion mutations in E2a. A deletion of 1.3 kb removing codons 40 to 471 of the 529 amino acids of E2a was introduced into plasmids for preparation of viruses and vectors. An Ad5 virus with disruption of the E1 gene and deletion of E2a grew on 293-C2 cells but not on 293 cells. Vectors with E1 and E2a deleted expressing *Escherichia coli* β-galactosidase or human α_1-antitrypsin were prepared and expressed the reporter genes after intravenous injection into mice. This vector system retains sequences in common between the complementing cell line and the vectors, including 3.4 kb upstream and 1.1 kb downstream of the deletion. These vectors have potential advantages of increased capacity for insertion of transgene sequences, elimination of expression of E2a, and possibly reduction in expression of other viral proteins. Although the titers of the vectors with deleted are about 10- to 30-fold below those of vectors with E2a wild-type regions, the former vectors are suitable for detailed studies with animals to evaluate the effects on host immune responses, on duration of expression, and on safety.

Vectors derived from human adenoviruses have many advantages for somatic gene therapy, including high titer, efficient gene transfer, limited pathological potential, and feasibility for delivery in vivo, as has been reviewed previously (4, 7, 12, 44). Adenovirus vectors have been used in animals for in vivo delivery into the blood stream, into the airway, and into a variety of tissues including muscle, brain, and tumors (6, 26, 34, 36). Adenoviruses have been shown to be safe when used as vaccines (11, 45) and are being used extensively in early clinical trials aimed at treatment of genetic disease (39) and cancer (26).

Most of the adenovirus vectors initially developed for gene therapy—often referred to as first-generation vectors—contained deletions in the essential E1 region and sometimes contained deletions in the E3 region as well (4, 5, 23). These vectors can be propagated in 293 cells, a human embryonic kidney cell line which complements the E1a and E1b functions (25). These first-generation adenovirus vectors are toxic and even lethal when given to animals in high doses (6, 8) and have caused significant inflammatory responses in gene therapy trials (13). First-generation adenovirus vectors typically result in limited duration of transgene expression, i.e., 2 to 6 weeks, although prolonged expression has been observed when vectors have been delivered to neonatal (43) or immunodeficient (51, 54) animals and occasionally when they have been delivered to normal adult animals (3).

The toxicity of adenovirus vectors may be related to the direct effect of the vector particle or to low levels of replication or expression of the virus after entry. To the extent that replication or expression of the virus may contribute to toxicity, the development of more-attenuated vectors might be helpful, and multiple efforts to develop refinements in adenovirus vectors have been reported. Vectors with a temperature-sensitive mutation in the E2 gene are reported to prolong transgene expression (14). A vector derived from adenovirus type 2 (Ad2) was modified by deleting portions of the E4 region and retaining expression of open reading frame 6 (ORF 6) (2). This vector can be grown in 293 cells, since the E4 regions deleted are not essential for replication of the virus in cultured cells. There are two reports of vectors with deletion of the E4 gene and development of cell lines complementing the E4 function using transfection of 293 cells (30, 50). In one case, the cell line also expresses protein IX in *trans*, allowing a larger deletion of the E1 region (30). Both complementing cell lines use inducible promoters for expression of E4. There are also two reports of adenovirus vectors with all of the viral coding sequences deleted (17, 29). These vectors require the use of a helper virus for propagation of the vector. Development of complementing cells that express E2b (DNA polymerase) was reported recently (1), and this may facilitate the use of viral vectors defective in E2b. At present, there are limited published data documenting that these "second-generation" vectors are less toxic when used in vivo (20).

It has been suggested that host responses to low levels of expression of viral proteins may limit the duration of expression of adenovirus vectors (8, 13, 51, 53) and that vectors with a temperature-sensitive mutation in the E2a region demonstrate prolonged expression (14, 20, 52), although the latter effect was minimal in some experiments (16). The host immune response to viral proteins synthesized from vectors has received considerable attention, and many animals develop a dose-dependent cellular inflammatory reaction (8, 13, 51, 53). Synthesis of viral DNA and expression of viral proteins occur with vectors with E1 deleted (32, 51). Blunting of the host immune response appears to have been an important determi-

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TABLE 1. Plasmids, viruses, and viral vectors

Plasmid, virus, or vector	Description	Reference(s)
Plasmids		
pFG140	Insertion of 2.21 kb in E1; dl309 in E3	22, 24
pFG140ΔE2	pFG140 with deletion in E2a	This report
pJM17	Insertion of 4.37 kb in E1; dl309 in E3	31
pBHGE3	Deletion of packaging signal and E1	5
pΔψE1E2	pBHGE3 with deletion in E2a	This report
pBHG11	Deletion of packaging signal, E1, and E3	5
pΔψE1E2E3	pBHG11 with deletion in E2a	This report
pXCJL.1/CMV/nls.lacZ	Shuttle for producing β-Gal vectors[a]	18
pPGK-hAAT.1	Shuttle for producing AAT vectors[b]	27
Pol2sneobpA	Contains neomycin resistance cassette	41
pBZ20	Contains 5.87-kb E2 fragment in pNEB193	This report
Viruses		
Adenovirus H5ts125	Ad5 with E2a temperature-sensitive mutation	15, 19
Adenovirus dl802	Ad5 with deletion in E2a	40
AdFG140	Virus derived from pFG140	This report
AdFG140ΔE2	Virus with E2a deleted derived from pFG140ΔE2	This report
Vectors		
AdHCMVsp1lacZ	β-Gal-expressing vector with E1 deleted	35
AdβgalΔE1	β-Gal-expressing vector with E1 deleted; derived from PBHGE3 and pXCJL.1/CMV/nls.lacZ	This report
AdβgalΔE1E2	β-Gal-expressing vector with E1 and E2 deleted; derived from pΔψE1E2 and pXCJL.1/CMV/nls.lacZ	This report
AdhAATΔE1	AAT-expressing vector with E1 deleted; derived from pBHGE3 and pPGK-hAAT.1	This report
AdhAATΔE1E2	AAT-expressing vector with E1 and E2a deleted; derived from pΔψE1E2 and pPGK-hAAT.1	This report

[a] β-Gal, β-galactosidase.
[b] AAT, α_1-antitrypsin.

nant of long-term expression in many experiments when vectors were administered to newborn mice, to immunodeficient mice, or to pharmacologically immunosuppressed mice (14, 43, 51, 54).

This report focuses on vectors with deletion of the E2a gene, which encodes a DNA-binding protein (DBP). The DBP is essential for the elongation and efficient initiation of viral DNA replication (42, 46, 47). It stimulates the transcription of adenovirus early and late genes and plays a role in the assembly of virus particles (37). The DBP stimulates the adenovirus major late promoter to a greater degree than does the E1a transactivator protein, suggesting that the DBP plays a central role in activation of the late promoter (10). Given these properties of the E2a gene and reports that a temperature-sensitive mutation in the gene prolongs transgene expression, development of an adenovirus vector with a deletion rather than the temperature-sensitive mutation of E2a would be of interest. It is reasonable to anticipate that a deletion of the E2a gene in an adenovirus vector would further reduce viral DNA replication and protein synthesis; it can be hypothesized that this might decrease the toxicity of the vector, decrease the immune response to the virus infection, and increase the duration of the transgene expression. On the basis of the strategy of Bett et al. for vectors with E1 deleted (5), we have developed an adenovirus vector system with E1 and E2a deleted which includes the construction of vectors with E1 and E2a deleted and the development of an E1- and E2a-complementing cell line capable of supporting growth of these vectors.

MATERIALS AND METHODS

Plasmids, viruses, and vectors. Brief descriptions and references for plasmids, viruses, and vectors obtained elsewhere are provided in Table 1. Plasmid pFG140 contains a relatively small insertion of plasmid sequence in the E1 region of the Ad5 genome and is infectious in 293 cells (22, 24). Plasmid pJM17 (31) is identical to pFG140 except that it contains a larger insert in the E1 region which prevents efficient packaging of virus particles. Plasmids pBHGE3 and pBHG11 have deletions of part of the E1 region, including the essential viral packaging signal; pBHG11 also carries a deletion in the E3 region, while pBHGE3 is wild type for E3 (5). Plasmids pXCJL.1/CMV/nls.lacZ (18) and pPGK-hAAT.1 (27) are adenovirus shuttle constructs containing expression cassettes for *E. coli* β-galactosidase (*lacZ*) and human α_1-antitrypsin (hAAT), respectively.

Adenovirus H5ts125 is a temperature-sensitive mutant containing a single base substitution in the E2a gene. This mutant propagates in normal cells at 33°C but not at the restrictive temperature (39°C) (15, 19). Adenovirus dl802 is a mutant that contains a 242-bp deletion in the E2a ORF resulting in absence of detectable E2a protein (40). AdHCMVsp1lacZ (AdlacZ) is an adenovirus vector with E1 deleted and with the *lacZ* gene as a reporter (35). Propagation of adenovirus vectors was carried out as described previously (23).

Construction of E2a expression cassette and cell lines. For the development of E1- and E2a-complementing cell lines, we constructed a plasmid (pBZ20) that expresses both E2a and neomycin resistance (Fig. 1). To construct pBZ20, the 5,870-bp *Eco*RI-*Bam*HI fragment containing the E2a gene with its own promoters (early and late) was removed from the Ad5 plasmid pFG140. This fragment was cloned into pNEB193 (New England Biolabs, Beverly, Mass.); a neomycin resistance cassette excised with *Xho*I from pol2sneobpA (41) also was inserted. Plasmid pBZ20 was transfected into 293 cells, which complement the E1 genes, by calcium phosphate precipitation (23). All cells were grown in MEMα (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah). Two days after transfection, the cells were selected in 250 μg of G418 per ml. The medium was changed every 3 to 5 days until neomycin-resistant colonies were visible. The colonies were cloned and screened for E2a complementing ability with adenovirus H5ts125. Analysis of expression of the E2a gene was performed by Patricia Berthelette (Genzyme, Framingham, Mass.) by immunostaining of cultured cells using a rabbit antibody to DBP (49). Complementing cell lines were characterized as described in Results.

Construction of plasmids with E1 and E2a deleted. In order to prepare vectors defective for E1 and E2a, a deletion of E2a was introduced into three plasmids (pFG140, pBHGE3, and pBHG11) containing the majority of the adenovirus genome for use as described in Results. As a first step in the construction of the vectors with E2a deleted, a *Bam*HI-*Xho*I fragment of 3.2 kb corresponding to 59.9 to 68.9 map units (m.u.) (m.u. and nucleotide sequence coordinates refer to Ad5 [GenBank accession no. M73260]) of the adenovirus genome was subcloned from pFG140 (Fig. 1). Next, a segment of 1,294 bp (bp 22618 to 23912) of the E2a ORF was removed by using the *Eco*RV (62.8 m.u.) and *Sma*I (66.4 m.u.) sites shown in Fig. 1. Because there are multiple *Xho*I sites in pFG140, the deleted *Bam*HI-*Xho*I fragment was reintroduced into pFG140 in multiple steps. First, the deleted *Bam*HI-*Xho*I fragment was cloned into the larger *Bam*HI-*Eco*RI fragment (59.9 to 75.9 m.u.) of pFG140 which contains unique *Bam*HI

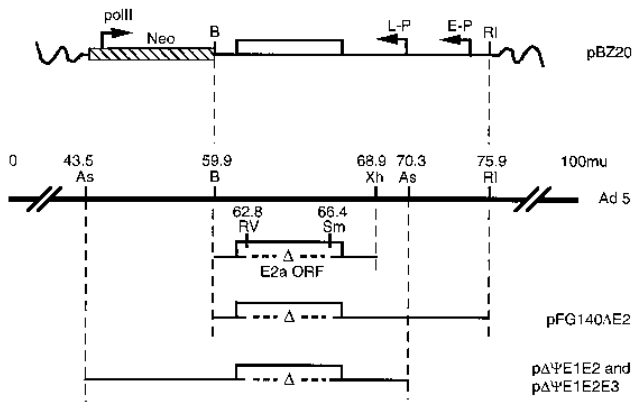

FIG. 1. Depiction of the Ad5 regions used to develop the E2a-complementing cell line and to construct vectors with E2a deleted. The adenovirus fragment in plasmid pBZ20 containing the E2a ORF and two promoters is shown at the top. The wild-type segment of Ad5 from pFG140 is also shown, with the *Bam*HI-*Xho*I fragment used as an intermediate in the preparation of the deletion from *Eco*RV to *Sma*I immediately below it. The *Bam*HI-*Eco*RI fragment transferred into pFG140ΔE2 also is shown. The *Asc*I fragment transferred from pFG140ΔE2 to pΔψE1E2 and pΔψE1E2E3 is shown at the bottom of the figure. As, *Asc*I; B, *Bam*HI; Xh, *Xho*I; RI, *Eco*RI; RV, *Eco*RV; Sm, *Sma*I.

and *Eco*RI sites. The *Bam*HI-*Eco*RI fragment was then substituted into pFG140 to yield pFG140ΔE2 (Fig. 1). The deleted E2a gene in pFG140ΔE2 was then introduced into other adenovirus genomic plasmids, i.e., pBHGE3 and pBHG11, by replacement of the *Asc*I fragment between 43.5 and 70.3 m.u. to produce plasmids pΔψE1E2 and pΔψE1E2E3, respectively (Fig. 1). These adenovirus genomic plasmids containing deletions in E2a were then used to develop vectors expressing reporter genes.

Construction of adenovirus vectors containing reporter genes. Development of infectious vectors from the adenovirus genomic plasmids was conducted essentially as described previously and involved cotransfection of the adenovirus genomic plasmid with a shuttle plasmid (23) with some modifications. All the plasmid DNAs used in transfection were purified with plasmid columns from Qiagen, Inc. (Chatsworth, Calif.) rather than with CsCl banding. For transfection, 293-C2 (C2) cells (an E1- and E2a-complementing cell line described in Results) were used to prepare vectors with E2a wild-type sequences or with deletions in E2a. In a standard transfection, about 2×10^5 C2 cells in 2 ml of medium were added to each well in a six-well plate. The next day the cells were transfected at about 60 to 80% confluence. Cotransfection was performed with 20 μg of pBHGE3 and 20 μg of either pXCJL.1/CMV/nls.lacZ or pPGK-hAAT.1. Fifty microliters of 2.5 M $CaCl_2$ was added dropwise to the plasmid DNA in 1 ml of HEBS buffer (23). After 30 min at room temperature to precipitate the DNA, 0.2 ml of DNA suspension was added to each of five wells in a six-well plate (leaving one well as a negative control). Plates were incubated for 4 to 16 h, the medium was removed, and 4 ml of fresh medium was added. Medium was changed every 3 to 5 days, and the plates were maintained until cytopathic effect (CPE) was evident in about 10 days. The method used to develop vectors with E2a deleted was essentially the same as that described above except that plasmid pΔψE1E2, with E2a deleted, was substituted for pBHGE3. To provide transient higher levels of E2a expression, 5 μg of an E2a-expressing plasmid (either pBHGE3 or pBH20) was included in the transfection mixture (see Results). The CPE for vectors with E2a deleted was evident in about 20 days or longer. After development of CPE, viruses were isolated by plaque purification.

Virus purification, titer, and structure determinations. Plaque purification and assay were performed in six-well plates as described previously (23) with 293 cells or C2 cells as indicated below. Plaques appeared 3 to 5 days after infection with vectors with E1 deleted and with wild-type E2a. For viruses with deletions of both E1 and E2a, plaques were typically smaller and required 1 or more days longer relative to those for corresponding viruses with E1 deleted and with wild-type E2a. Purified virus was analyzed for the presence and expression of the reporter genes by PCR, by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining for expression of β-galactosidase, and by enzyme-linked immunosorbent assay (27) for production of hAAT in culture medium.

PCR analysis of virus with E2a deleted. For a rapid PCR assay, 3 μl of culture medium from cells demonstrating CPE after viral infection was added to a PCR mixture containing AmpliTaq DNA polymerase under conditions recommended by the supplier (Perkin-Elmer Cetus, Norwalk, Conn.). Alternatively, viral DNA for PCRs was prepared from purified virus by phenol extraction and ethanol precipitation. Two pairs of PCR primers, i.e., 551 (5′-CCGGCAAGTCTTGCG GCATG) and 556 (5′-TAGCAGGTCGGGCGCCGATAT), located inside of the E2a ORF, and 731 (5′-AGTGCGCAGATTAGGAGCGC) and 732 (5′-GC CTATAGGAGAAGGAAATG), flanking the E2a ORF, were used in the reactions. PCR conditions were 94°C for 2 min followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

RESULTS

Development of an E1- and E2a-complementing cell line. Because the E2a gene product (DBP) is essential for adenovirus growth, propagation of adenovirus vectors with deletions in both E1 and E2a required development of a new complementing cell line capable of providing both gene products. To develop such a cell line, we constructed plasmid pBZ20, which included both the E2a gene under the control of the natural E2a promoters and an expression cassette for neomycin resistance (Fig. 1). This plasmid was used to transfect 293 cells, and neomycin-resistant cells were selected with G418. Over 100 neomycin-resistant colonies were picked and screened for E2a complementing ability with the H5ts125 virus. On 293 cells, this temperature-sensitive mutant replicated and produced CPE at the permissive temperature (33°C) but did not yield CPE at the restrictive temperature (39°C). H5ts125 virus did produce CPE at both 33 and 39°C in about half of the neomycin-resistant clones, providing evidence of complementation of the E2a gene.

Some complementing cell clones grew slowly and rounded up during culture, suggesting the possibility of toxic levels of E2a. Other cell clones, when infected with H5ts125 virus, required a longer time for development of CPE at 39°C than they did at 33°C, suggesting lower levels of E2a. Among the clones analyzed, one, i.e., C2, grew slowly at first but grew as well as the parent 293 cell line after several passages. Compared with other clones tested, C2 cells required the shortest time for development of CPE at the restrictive temperature after infection with H5ts125. The C2 cell line did not have any notable morphological changes compared with 293 cells (Fig. 2) and was capable of supporting plaque formation after infection with both H5ts125 (data not shown) and adenovirus dl802 with E2a deleted (Fig. 2).

The E2a-complementing ability of C2 cells should be due to the expression of the transfected sequences expressing E2a. The presence of the E2a sequences in C2 cells was confirmed by Southern hybridization of genomic DNA (Fig. 3). By use of restriction enzymes cutting within the plasmid sequence (*Eco*RV, *Bam*HI-*Xho*I, and *Bam*HI-*Eco*RI), the transfected sequences were demonstrated to be intact in C2 cells and absent in 293 cells. The C2 cell line has been cultured for over 1 1/2 years (up to 60 passages) in the absence of G418 without loss of E2a-complementing ability, but selection in G418 may be useful for maintaining complementing ability.

Expression of E2a in C2 cells. Expression of E2a in C2 cells was analyzed by reverse transcription-PCR, Northern (RNA) hybridization, and immunostaining. Reverse transcription-PCR and Northern blotting detected expression of E2a in C2 cells but not in parental 293 cells (data not shown). The levels of expression were less than those detected after productive infection of 293 cells with an E2a wild-type vector. Expression of the E2a gene product (DBP) also was analyzed with rabbit antibody to DBP (49); C2 cells showed intense nuclear immunofluorescence compared with an absence of staining in 293 cells, confirming the proper expression and nuclear localization of the E2a protein (Fig. 4).

Development of adenovirus plasmids with E1 and E2a deleted. The E2 genes are transcribed leftward in the adenovirus genome. The E2a ORF does not overlap with protein coding sequences for the late genes which are transcribed rightward,

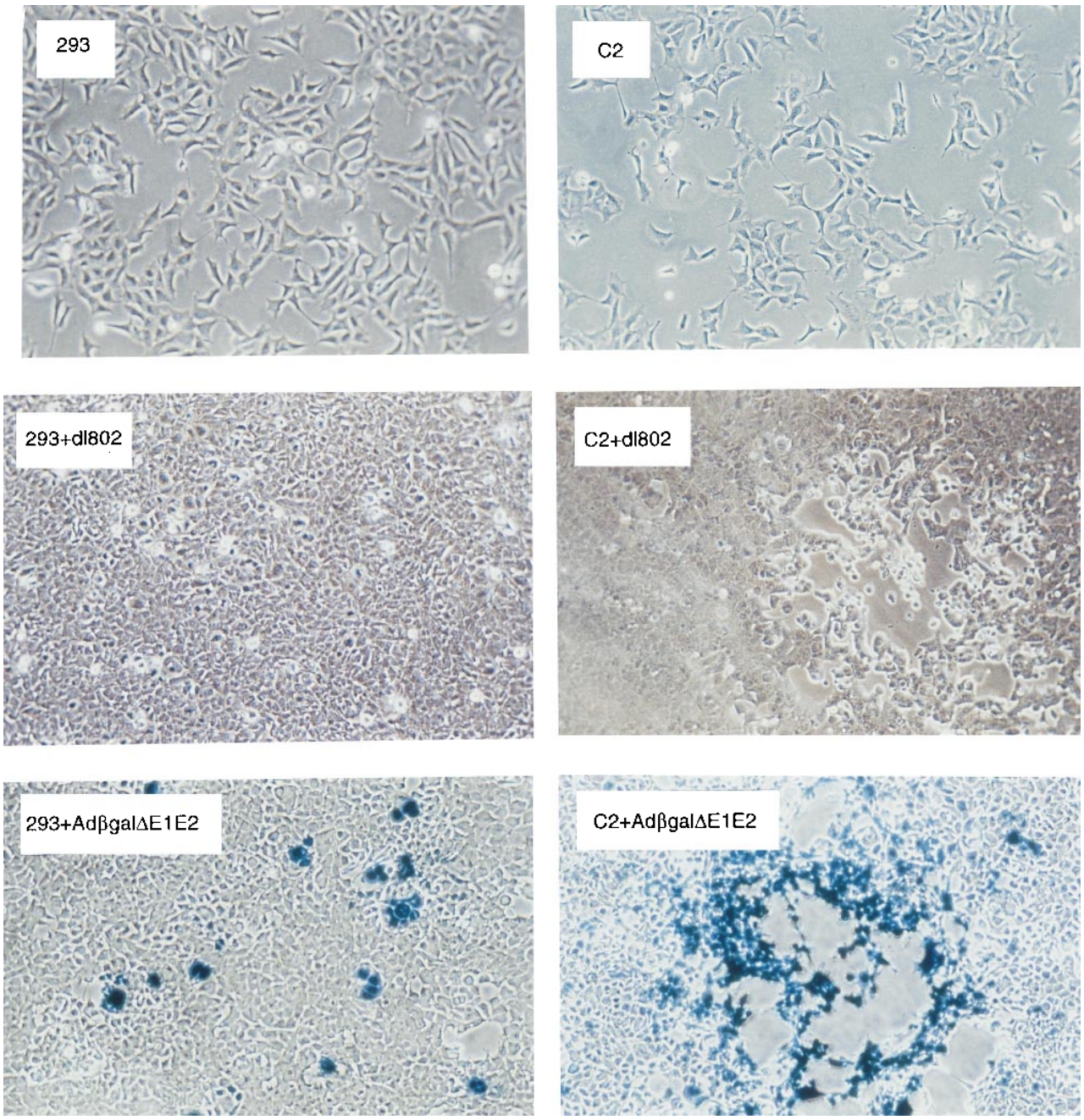

FIG. 2. Comparison of 293 cells and C2 cells with regard to morphology and plaque formation. The 293 cells and C2 cells were uninfected (top); infected with adenovirus dl802, with E2a deleted (middle); or infected with the vector expressing β-galactosidase, AdβgalΔE1E2, with E2a deleted (bottom). All microscopy is at a magnification of ×150.

and the poly(A) signal for the L3 gene and the splicing signal for the L4 gene are outside of this region (48). Therefore, deletion of the E2a ORF in the adenovirus vectors should not interrupt other essential genes. By deletion of 1,294 bp of the Ad5 E2a between the *Eco*RV and *Sma*I sites in the ORF (Fig. 1), we constructed three adenovirus plasmids with E2a deleted: pFG140ΔE2, pΔψE1E2, and pΔψE1E2E3. The deletion removes codons 40 to 471 of the 529-amino-acid ORF for E2a but does not encompass the 5′ or 3′ regions of the E2a gene (Fig. 1). Flanking the E2a deletion, two segments remain in common between the adenovirus DNA integrated into the C2 cells and the vectors with E2a deleted, i.e., 3,419 bp of sequence in the promoter region and 1,056 bp of sequence at the 3′ end of the E2a gene (Fig. 1).

The plasmids with deletions in E2 were derived by stepwise molecular procedures as described in Materials and Methods. Plasmid pFG140ΔE2 was derived from pFG140 and is suitable for preparing a virus with inserted sequences in E1 and a deletion in E2. Plasmid pΔψE1E2 was prepared from pBHGE3 and is suitable for preparing vectors with deletions of E1 and E2a. Plasmid pΔψE1E2E3 was prepared from pBHG11 and is useful for preparing vectors with E1, E2a, and E3 de-

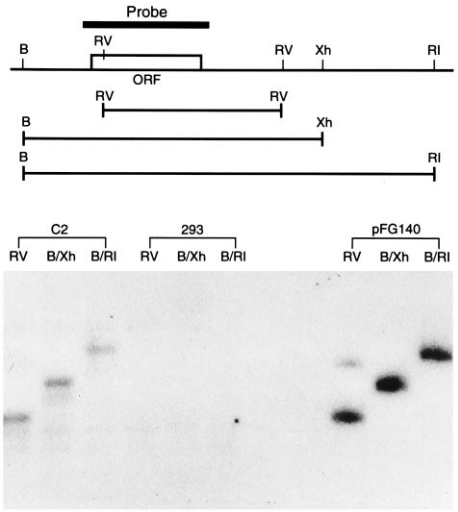

FIG. 3. Southern blot analysis of the E2a sequences in C2 genomic DNA. The probe was a PCR product covering the entire E2a ORF as depicted. The fragments expected after digestion with *Eco*RV, *Bam*HI-*Xho*I, or *Bam*HI-*Eco*RI are indicated. The expected fragments were observed in C2 cells and in the pFG140 plasmid but not in 293 cells. B, *Bam*HI; RV, *Eco*RV; Xh, *Xho*I; RI, *Eco*RI.

leted. All of these plasmids were documented to contain the appropriate deletions as exemplified in Fig. 5 by PCR analysis of pFG140ΔE2. The deletion of the E2a gene in these vectors was confirmed by restriction enzyme analysis with *Hin*dIII, *Asc*I, and *Eco*RI-*Bam*HI (data not shown).

Generation of virus with E2a deleted from plasmid pFG140ΔE2. Plasmid pFG140 contains all the required adenovirus genes except E1 and is infectious in 293 cells (24). When pFG140 was transfected into 293 cells, the plasmid was converted to an infectious virus designated AdFG140. Plasmid pFG140ΔE2 is identical to pFG140 except for the deletion in the E2a gene. Therefore, pFG140ΔE2 should not be infectious in 293 cells but should be infectious in C2 cells. The pFG140ΔE2 plasmid did prove to be infectious in C2 cells with CPE developing about 10 days after transfection. The resulting virus, designated AdFG140ΔE2, formed plaques on C2 cells but not on 293 cells. The AdFG140ΔE2 virus was confirmed to contain the E2a deletion by using PCR to compare it with AdlacZ, which contains a wild-type E2a region (Fig. 5). Primers within the deletion yielded no product, and primers flanking the deletion yielded a product of the expected shortened size. There was no evidence of contamination of the AdFG140ΔE2 virus with recombinant virus containing wild-type E2a sequences in this preparation by PCR analysis under conditions that were documented to detect wild-type sequences at a level of 1 ppm in mixing experiments.

By using the AdFG140ΔE2 virus, the relative complementing abilities of many of the clones which were screened initially were retested. The C2 cell line was confirmed to provide the best complementing capacity. In order to compare the growth of a virus with E2a deleted with that of a comparable virus with wild-type E2a region, the AdFG140 and AdFG140ΔE2 viruses were used to infect 293 cells and C2 cells at a multiplicity of infection of 1 (Fig. 6). Aliquots of culture medium were taken every 12 h for analysis by plaque assay. In cultures infected with the virus containing the wild-type E2a region (AdFG140), the titer with C2 cells was slightly higher than that with 293 cells, indicating that the E1-complementing ability of C2 cells was not diminished (Fig. 6). The virus with E2a deleted

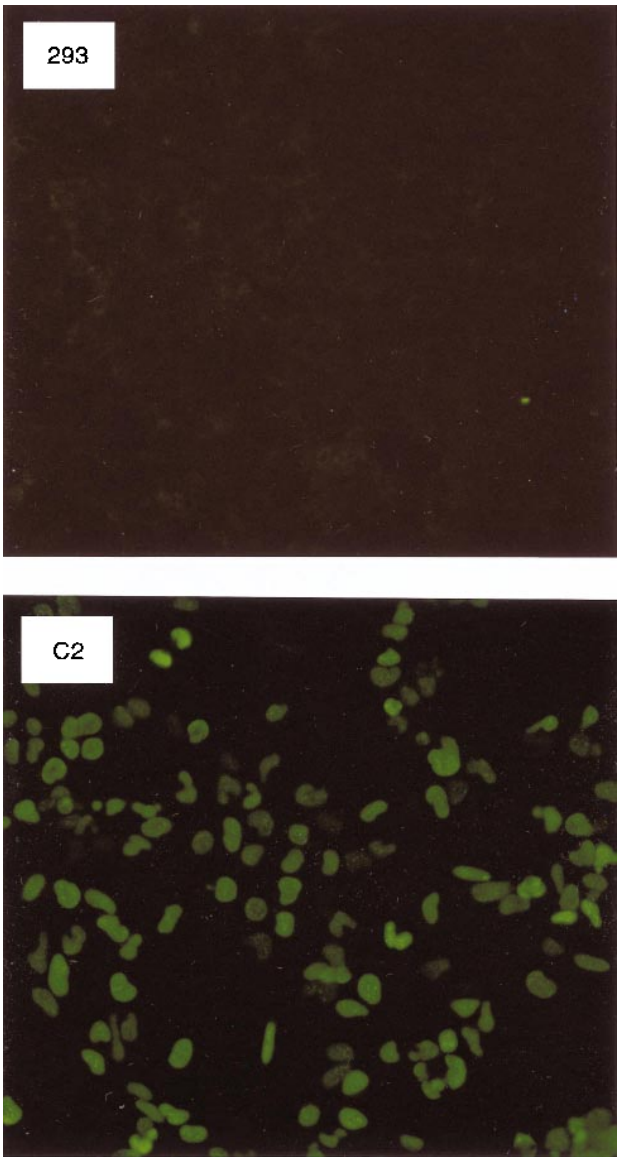

FIG. 4. Immunofluorescent staining of E2a in 293 cells and C2 cells. The E2a gene product was detected by immunofluorescence as described in Materials and Methods. Cells were plated at equal densities, but no immunostaining was seen in 293 cells whereas intense nuclear staining was seen in C2 cells.

(AdFG140ΔE2) did not grow on 293 cells but did produce substantial virus on C2 cells (Fig. 6). The titer of AdFG140ΔE2 was 10- to 30-fold lower than that of AdFG140 when both were grown on C2 cells (Fig. 6). This might suggest that the E2a gene product is limiting during a productive infection.

Introduction of expression cassettes into adenovirus vectors with E1 and E2a deleted. Plasmids pΔψE1E2 and pΔψE1E2E3, as well as their parental (E2a wild type) plasmids pBHGE3 and pBHG11, are not infectious in complementing cell lines, because of a deletion of the adenovirus packaging signal (5). This characteristic is useful for introducing exogenous genes into these adenovirus vectors. Like their parental plasmids, pΔψE1E2 and pΔψE1E2E3 can be used to prepare infectious vectors with expression cassettes via homologous recombination with a shuttle plasmid containing the packaging signal and an exogenous gene.

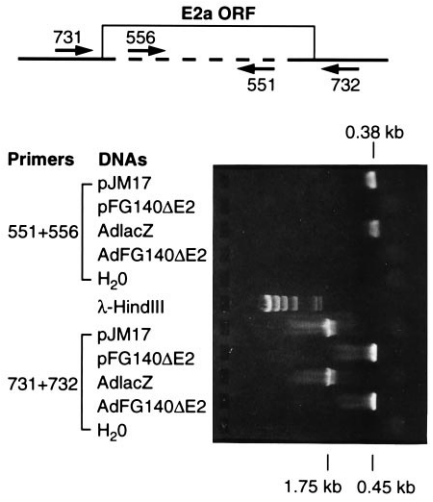

FIG. 5. PCR analysis of the deleted E2a region in plasmids and viruses. Primers within and flanking the E2a ORF are indicated at the top. Primers within the deletion did not yield any PCR product for the pFG140ΔE2 and AdFG140ΔE2 plasmid and virus, respectively, while the appropriate product was seen for the control plasmid and virus. The molecular weight markers and water blanks are indicated. The origin of the gel is to the left.

Two separate reporter genes, that for hAAT and *E. coli lacZ*, were introduced into an adenovirus vector with E1 and E2a deleted. The DNA of the shuttle plasmid pXCJL.1/CMV/nls.lacZ (18) or pPGK-hAAT.1 (27) was cotransfected with DNA of pΔψE1E2 into C2 cells. As a control, each of the two shuttle plasmids was also cotransfected with pBHGE3 (the wild-type E2a-containing counterpart of pΔψE1E2) into C2 cells to generate vectors with E1 deleted differing from the vectors with E2a deleted only in the E2a region. After cotransfection, infectious vectors with E1 deleted expressing the reporter genes were obtained from the cell culture transfected with pBHGE3 and the shuttle plasmids. However, no viral vectors with E1 and E2a deleted were obtained in this way despite several attempts.

Although there were various possible explanations for the difficulties in recovering the vectors with E1 and E2a deleted, we tested the possibility that transient expression of higher levels of E2a might facilitate recovery of the vectors. In initial efforts to increase the expression of E2a, three plasmids were cotransfected: pBHGE3 to provide transient expression of E2a and pΔψE1E2 and the shuttle plasmid pXCJL.1/CMV/nls.lacZ in the hope that these two plasmids would recombine to yield the desired vector expressing *lacZ*. After cotransfection with the three plasmids, infectious viruses expressing *lacZ* were detected in cell cultures demonstrating CPE. The E2a region was analyzed by PCR, and a mixture of sequences with wild-type E2a and sequences with E2a deleted was found. Since both the plasmid with wild-type E2a (pBHGE3; 5 μg) and the plasmid with E2a deleted (pΔψE1E2; 20 μg) may recombine with the shuttle plasmid, it was anticipated that both vectors with wild-type E2a and vectors with E2a deleted expressing *lacZ* would occur. Individual plaques were picked and analyzed by PCR for the presence or absence of the E2a region. After plaques were identified as vectors with E2a deleted and with no wild-type E2a sequences, three additional rounds of plaque purification were performed before the vector was used for further analysis and for large-scale preparations. These initial stocks of each vector were found to be free of contamination with wild-type E2 sequences when analyzed by PCR amplification of the deleted region as described above. The vector with E1 and E2a deleted expressing *lacZ* was designated AdβgalΔE1E2. We concluded that transient expression of the E2a gene from plasmid pBHGE3 may increase the frequency of homologous recombination between the adenovirus genomic plasmid and the shuttle plasmid. Subsequently, plasmid pBZ20, which contains a smaller fragment of adenovirus DNA expressing E2a, was used instead of pBHGE3 as a source of transient expression of E2a, and the other vector with E1 and E2a deleted, expressing hAAT, was recovered and designated AdhAATΔE1E2.

When AdβgalΔE1E2 was used to infect C2 cells, plaques formed in 5 to 6 days (Fig. 2). When 293 cells were infected with AdβgalΔE1E2, the *lacZ* gene was expressed in individual cells, resulting in staining with X-Gal, but no plaque formation was detected under these conditions (Fig. 2). Although AdβgalΔE1E2 was unable to form plaques in 293 cells, the vector could infect these noncomplementing cells and express the reporter gene.

In vivo expression using vectors with E2a deleted. In a large-scale preparation of adenovirus vectors for in vivo experiments, 16 triple-layer flasks (500 cm^2 per flask) were used. By DNA measurement, the yield of vectors with wild-type E2a (AdβgalΔE1 and AdhAATΔE1) was found to be about 10^{12} particles per preparation on the basis of optical density determination of DNA, and the yield of vectors with E2a deleted (AdβgalΔE1E2 and AdhAATΔE1E2) was about 10^{11} particles. The ratio of the number virus particles as determined by DNA measurement to PFU was generally greater for preparations with E2a deleted than for preparations with wild-type E2a, but the PFU titer of vectors with E2a deleted may have been underestimated in some cases because the vectors with E2a deleted require a longer period to form plaques and the size of plaques is generally smaller than that for vectors with wild-type E2a. The AdβgalΔE1E2 virus was used for intravenous injection into mice. Injection of 8×10^7 PFU resulted in nuclear targeted expression of β-galactosidase in a substantial fraction of hepatocytes (Fig. 7). Intravenous injection of the AdhAATΔE1E2 vector into mice resulted in expression of

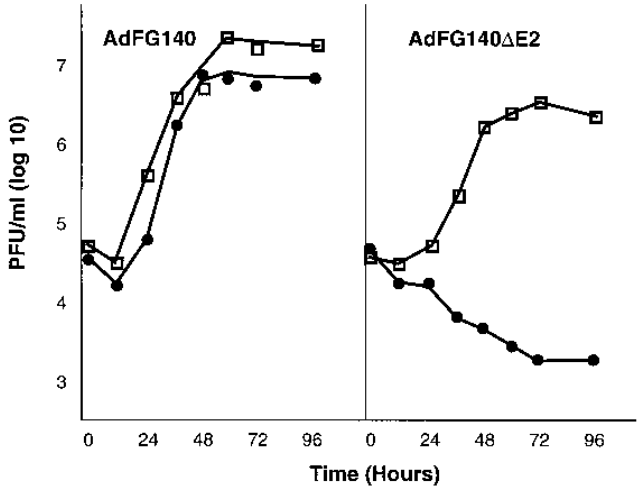

FIG. 6. Growth of viruses with wild-type E2a and with E2a deleted on 293 cells and C2 cells. Growth of the virus with wild-type E2a, AdFG140, is shown on the left, and growth of the virus with E2a deleted, AdFG140ΔE2, is shown on the right. Open squares indicate growth on C2 cells, and solid circles indicate growth on 293 cells. Medium was collected at the times indicated, and plaque assays were performed with C2 cells.

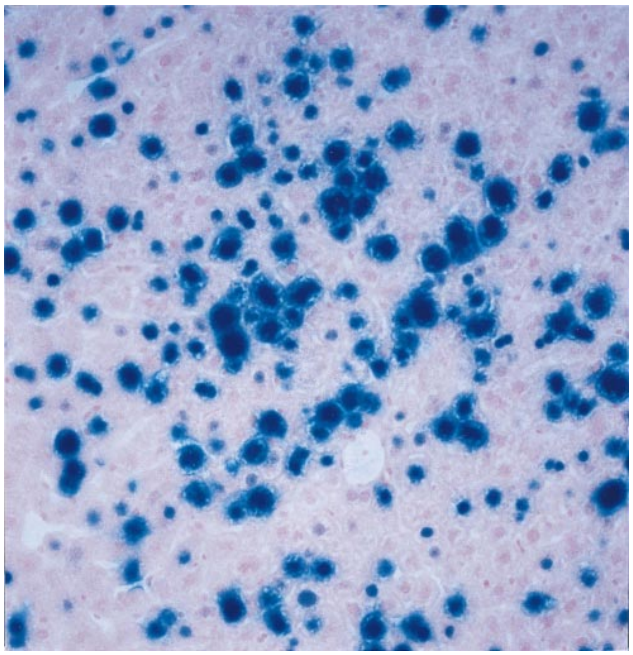

FIG. 7. Expression of β-galactosidase in mouse hepatocytes using a vector with E1 and E2 deleted. A C57BL/6 mouse was injected intravenously with 8×10^7 PFU of AdβgalΔE1E2. The animal was sacrificed after 4 days, and liver tissue was stained for expression of β-galactosidase.

hAAT in the plasma at levels similar to that observed with vectors with wild-type E2a at 2 to 3 days after injection (data not shown). There was no evidence of wild-type E2a sequences in the preparations used for these in vivo injections as tested by PCR as described above. These results indicate that these vectors with E2a deleted are suitable for use in vivo.

DISCUSSION

We have developed an adenovirus vector system with E1 and E2a deleted, building upon the previous availability of 293 cells and vectors with E1 deleted. The E1- and E2-complementing cell line C2 was developed by transfection of the E2a gene under the control of its own promoters into 293 cells. The resulting C2 cell line is very stable, with growth properties indistinguishable from those of the parental 293 cells. Plasmids with deletions of 1.3 kb of the ORF of E2a were developed in order to obtain adenovirus vectors with various expression cassettes. The pΔψE1E2E3 plasmid was derived from pBHGE3 to obtain an adenovirus genomic plasmid with E1 and E2a deleted. The pΔψE1E2 plasmid was derived from pBHG11 to obtain a plasmid with E1, E2a, and E3 deleted. Plasmids pΔψE1E2 and pΔψE1E2E3 lack the packaging signal and are noninfectious when transfected into complementing cells, making them suitable for rescue of expression vectors using appropriate shuttle plasmids as described previously (5). Plasmid pFG140ΔE2 was derived by introducing the E2 deletion into pFG140 and was shown to be infectious in C2 cells; this plasmid can be used as a control in DNA transfection during vector development. The viruses with E1 and E2 deleted developed in this study can replicate and plaque on C2 cells.

It was reported previously that E2-complementing cell lines can be difficult to develop because the product of the E2a gene is toxic to cells in culture (28). A cell line complementing E2a was developed by using the mouse mammary tumor virus (MMTV) promoter with induction of expression with dexamethasone (9, 28). When we transfected 293 cells with the E2a cassette under the control of the strong constitutive EF-1α promoter (33), the cell clones appeared to be unstable (data not shown). With use of the viral fragment containing the E2a ORF and the two natural promoters in the C2 cell line, it is expected that some level of E2a expression would occur, since the E1 production of the 293 or C2 cells would constitutively activate the E2a promoters. During the selection of clones complementing E2a, the C2 clone initially grew quite slowly. After several passages, C2 cells grew well and complemented the E2a function efficiently. The stable C2 cell line may have resulted from some selection in response to toxicity of E2a expression. The somewhat lower titers achieved with vectors with E2a deleted compared with vectors with wild-type E2 suggest that the E2a protein is limiting during production of vectors. It is possible that the level of expression of E2a in C2 cells is increased during infection by further activation of the E2a promoters, since it is known that adenovirus E4-6/7 proteins can transcriptionally activate the E2a gene by stimulating the binding of E2F to the promoters (38).

When with E1 and E2 deleted, plasmid pΔψE1E2, and a shuttle plasmid were cotransfected into C2 cells to introduce reporter genes into the adenovirus vector by homologous recombination, it was difficult to generate infectious vectors. This was not the case when pBHGE3, with wild-type E2a, and the shuttle plasmid were used. The difference observed may be due to the different levels of E2a gene product, DBP, in the transfected cells. In C2 cells transfected with a plasmid containing the wild-type E2a sequence, there is the opportunity for increased expression of E2a from the plasmid. This increase in DBP level might increase the potential for replication of viral DNA and could affect the probability of homologous recombination between the genomic plasmid and the shuttle plasmid. When a third plasmid containing a functional E2a gene was added in the cotransfections, two adenovirus vectors expressing reporter genes and carrying both E1 and E2a deletions were obtained after previous unsuccessful efforts, suggesting that the level of E2a expression in C2 cells may limit the efficiency of generating vectors by homologous recombination.

Although wild-type sequences for the E2 region were not detectable in initial stocks of plaque-purified virus, we did observe the appearance of wild-type E2a sequences in some vector preparations after multiple passages of vectors with E2a deleted in C2 cells. We presume that this is due to recombination between the homologous sequences in the vectors and the C2 cells; the common regions include 3.4 kb upstream and 1.1 kb downstream flanking the E2 region (Fig. 1). We are attempting to further modify the vector system with E1 and E2a deleted by totally deleting the E2a ORF in the vectors and shortening the viral fragment used for complementation. We are also exploring the use of an inducible promoter for expression of E2a in order to improve the yield for vector production.

The vectors with E1 and E2 deleted have several potential advantages over vectors with E1 deleted. With the deletion of 1.3 kb in the E2a ORF, the maximal packaging capacity is increased from a 8.3 kb in pBHG11 (5) to approximately 9.6 kb in pΔψE1E2E3. The presence of two deletions in noncontiguous essential regions should substantially reduce the probability of developing replication-competent adenovirus when vectors are packaged in complementing cell lines. There is evidence that vectors with the H5ts125 mutation in the E2a gene demonstrate increased duration of expression in vivo (52), although this effect is not always significant (16); it is hypothesized that this difference is related to decreased expression of viral proteins and decreased host response to the

vectors. It might be expected that vectors with an E2a deletion and more severe deficiency of E2a function would provide further advantages in regard to duration of expression in vivo. Deletion of the E2a ORF also eliminates a gene product which is thought to be toxic to host cells and has the potential to elicit inflammatory and cytotoxic T-cell responses. Vectors with an E2a deletion might demonstrate reduced viral DNA replication when used for gene therapy in vivo, but there are few data in this regard and it is unclear if reduced replication would be advantageous or deleterious in terms of duration of expression and safety. These features of vectors with E2 deleted may contribute to greater safety for gene therapy and may increase the duration of transgene expression.

The C2 complementing cell line and multiply deleted vectors developed here may have other research applications. For example, cell lines expressing multiple adenovirus genes may be useful for preparing adeno-associated virus vectors. However, the primary purpose in developing these vectors was to determine if they would provide greater safety and efficacy for gene therapy in vivo. Extensive experimentation in animals will be required to draw proper conclusions in this regard. As reviewed in the introduction, many attempts to improve adenovirus vectors have been reported, but most of the resulting vectors remain of potential but incompletely documented value. Complementing cell lines which eliminate overlap between the complementing DNA sequences and the vector are desirable and should reduce the potential for appearance of replication-competent adenovirus. Since some viral proteins are toxic to cells, many attempts to develop complementing cell lines have utilized inducible promoters for expression of the complementing sequences. At this time, we believe that it is extremely important to directly compare various first- and second-generation adenovirus vectors, particularly to address two questions, i.e., whether the vectors provide decreased toxicity and whether they are associated with increased duration of transgene expression. The vector system with E1 and E2 deleted reported here could be improved in a variety of ways, including elimination of overlapping sequences between the complementing cell line and the vector and improvement of viral titer, perhaps utilizing an inducible promoter. However, these vectors should be adequate to determine whether vectors with E2 deleted are associated with reduced toxicity and/or increased duration of transgene expression in vivo.

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ADDENDUM

After submission of the manuscript, similar independent work was reported (21).

REFERENCES

1. **Amalfitano, A., C. R. Begy, and J. S. Chamberlain.** 1996. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. Proc. Natl. Acad. Sci. USA **93:**3352–3356.
2. **Armentano, D., C. C. Sookdeo, K. M. Hehir, R. J. Gregory, J. A. St. George, G. A. Prince, S. C. Wadsworth, and A. E. Smith.** 1995. Characterization of an adenovirus gene transfer vector containing an E4 deletion. Hum. Gene Ther. **6:**1343–1353.
3. **Barr, D., J. Tubb, D. Ferguson, A. Scaria, A. Lieber, C. Wilson, J. Perkins, and M. A. Kay.** 1995. Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes in vivo: comparisons between immunocompetent and immunodeficient inbred strains. Gene Ther. **2:**151–155.
4. **Berkner, K. L.** 1988. Development of adenovirus vectors for the expression of heterologous genes. BioTechniques **6:**616–629.
5. **Bett, A. J., W. Haddara, L. Prevec, and F. L. Graham.** 1994. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. USA **91:**8802–8806.
6. **Boucher, R. C.** 1996. Current status of CF gene therapy. Trends Genet. **12:**81–84.
7. **Bramson, J. L., F. L. Graham, and J. Gauldie.** 1995. The use of adenoviral vectors for gene therapy and gene transfer in vivo. Curr. Opin. Biotechnol. **6:**590–595.
8. **Brody, S. L., M. Metzger, C. Danel, M. A. Rosenfeld, and R. G. Crystal.** 1994. Acute responses of non-human primates to airway delivery of an adenovirus vector containing the human cystic fibrosis transmembrane conductance regulator cDNA. Hum. Gene Ther. **5:**821–836.
9. **Brough, D. E., V. Cleghon, and D. F. Klessig.** 1992. Construction, characterization, and utilization of cell lines which inducibly express the adenovirus DNA-binding protein. Virology **190:**624–634.
10. **Chang, L., and T. Shenk.** 1990. The adenovirus DNA-binding protein stimulates the rate of transcription directed by adenovirus and adeno-associated virus promoters. J. Virol. **64:**2103–2109.
11. **Couch, R. B., R. M. Chanock, T. R. Cate, D. J. Lang, V. Knight, and R. J. Huebner.** 1963. Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract. Am. Rev. Respir. Dis. **88:**394–403.
12. **Crystal, R. G.** 1995. Transfer of genes to humans: early lessons and obstacles to success. Science **270:**404–410.
13. **Crystal, R. G., N. G. McElvaney, M. A. Rosenfeld, C. Chu, A. Mastrangeli, J. G. Hay, S. L. Brody, H. A. Jaffe, N. T. Eissa, and C. Danel.** 1994. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. Nat. Genet. **8:**42–51.
14. **Engelhardt, J. F., X. Ye, B. Doranz, and J. M. Wilson.** 1994. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. Proc. Natl. Acad. Sci. USA **91:**6196–6200.
15. **Ensinger, M. J., and H. S. Ginsberg.** 1972. Selection and preliminary characterization of temperature-sensitive mutants of type 5 adenovirus. J. Virol. **10:**328–339.
16. **Fang, B., H. Wang, G. Gordon, D. A. Bellinger, M. S. Read, K. M. Brinkhous, S. L. C. Woo, and R. C. Eisensmith.** 1996. Lack of persistence of $E1^-$ recombinant adenoviral vectors containing a temperature-sensitive E2A mutation in immunocompetent mice and dogs. Gene Ther. **3:**217–222.
17. **Fisher, K. J., H. Choi, J. Burda, S. Chen, and J. M. Wilson.** 1996. Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. Virology **217:**11–22.
18. **French, B. A., W. Mazur, N. M. Ali, R. S. Geske, J. P. Finnigan, G. P. Rodgers, R. Roberts, and A. E. Raizner.** 1994. Percutaneous transluminal in vivo gene transfer by recombinant adenovirus in normal porcine coronary arteries, atherosclerotic arteries, and two models of coronary restenosis. Circulation **90:**2402–2413.
19. **Ginsberg, H. S., J. F. Williams, W. H. Doerfler, and H. Shimojo.** 1973. Proposed nomenclature for mutants of adenoviruses. J. Virol. **12:**663–664.
20. **Goldman, M. J., L. A. Litzky, J. F. Engelhardt, and J. M. Wilson.** 1995. Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2a-defective recombinant adenoviruses: a preclinical toxicology study. Hum. Gene Ther. **6:**839–851.
21. **Gorziglia, M. I., M. J. Kadan, S. Yei, J. Lim, G. M. Lee, R. Luthra, and B. C. Trapnell.** 1996. Elimination of both E1 and E2a from adenovirus vectors further improves prospects for in vivo human gene therapy. J. Virol. **70:**4173–4178.
22. **Graham, F. L.** 1984. Covalently closed circles of human adenovirus DNA are infectious. EMBO J. **3:**2917–2922.
23. **Graham, F. L., and L. Prevec.** 1991. Manipulation of adenovirus vectors, p. 109E. *In* J. Murray (ed.), Methods in molecular biology. The Human Press, Inc., Clifton, N.J.
24. **Graham, F. L., J. Rudy, and P. Brinkley.** 1989. Infectious circular DNA of human adenovirus type 5: regeneration of viral DNA termini from molecules lacking terminal sequences. EMBO J. **8:**2077–2085.
25. **Graham, F. L., and J. Smiley.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36:**59–72.
26. **Herrmann, F.** 1995. Cancer gene therapy: principles, problems, and perspectives. J. Mol. Med. **73:**157–163.
27. **Kay, M. A., F. Graham, F. Leland, and S. L. Woo.** 1995. Therapeutic serum concentrations of human α1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. Hepatology **21:**815–819.
28. **Klessig, D. F., D. E. Brough, and V. Cleghon.** 1984. Introduction, stable integration, and controlled expression of a chimeric adenovirus gene whose product is toxic to the recipient human cell. Mol. Cell. Biol. **4:**1354–1362.

29. **Kochanek, S., P. R. Clemens, K. Mitani, H.-H. Chen, S. Chan, and C. T. Caskey.** 1996. A new adenoviral vector: replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase. Proc. Natl. Acad. Sci. USA **93:**5731–5736.
30. **Krougliak, V., and F. L. Graham.** 1995. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. Hum. Gene Ther. **6:**1575–1586.
31. **McGrory, W. J., D. S. Bautista, and F. L. Graham.** 1988. A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. Virology **163:**614–617.
32. **Mittereder, N., S. Yei, C. Bachurski, J. Cuppoletti, J. A. Whitsett, P. Tolstoshev, and B. C. Trapnell.** 1994. Evaluation of the efficacy and safety of *in vitro*, adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA. Hum. Gene Ther. **5:**717–729.
33. **Mizushima, S., and S. Nagata.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. **18:**5322.
34. **Morgan, J. E.** 1994. Cell and gene therapy in Duchenne muscular dystrophy. Hum. Gene Ther. **5:**165–173.
35. **Morsy, M. A., E. L. Alford, A. Bett, F. L. Graham, and C. T. Caskey.** 1993. Efficient adenoviral-mediated ornithine transcarbamylase expression in deficient mouse and human hepatocytes. J. Clin. Invest. **92:**1580–1586.
36. **Neve, R. L.** 1993. Adenovirus vectors enter the brain. Trends Neurosci. **16:**251–253.
37. **Nicolas, J. C., P. Sarnow, M. Girard, and A. J. Levine.** 1983. Host range temperature-conditional mutants in the adenovirus DNA binding protein are defective in the assembly of infectious virus. Virology **126:**228–239.
38. **O'Connor, R. J., and P. Hearing.** 1994. Mutually exclusive interaction of adenovirus E4-6/7 protein and the retinoblastoma gene product with internal domains of E2F-1 and DP-1. J. Virol. **68:**6848–6862.
39. **O'Neal, W. K., and A. L. Beaudet.** 1994. Somatic gene therapy for cystic fibrosis. Hum. Mol. Genet. **3:**1497–1502.
40. **Rice, S. A., and D. F. Klessig.** 1985. Isolation and analysis of adenovirus type 5 mutants containing deletions in the gene encoding the DNA-binding protein. J. Virol. **56:**767–778.
41. **Soriano, P., C. Montgomery, R. Geske, and A. Bradley.** 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell **64:**693–702.
42. **Stillman, B. W.** 1985. Biochemical and genetic analysis of adenovirus DNA replication in vitro, p. 1–27. *In* J. K. Setlow and A. Hollaender (ed.), Genetic engineering principles and methods. Plenum Press, New York.
43. **Stratford-Perricaudet, L. D., I. Makeh, M. Perricaudet, and P. Briand.** 1992. Widespread long term gene transfer to mouse skeletal muscles and heart. J. Clin. Invest. **90:**626–630.
44. **Stratford-Perricaudet, L., and M. Perricaudet.** 1991. Gene transfer into animals: the promise of adenovirus. Hum. Gene Ther. **219:**51–61.
45. **Top, F. H., Jr., E. L. Buescher, W. H. Bancroft, and P. K. Russell.** 1971. Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7. J. Infect. Dis. **124:**155–160.
46. **Tsuji, M., P. C. van der Vliet, and G. R. Kitchingman.** 1991. Temperature-sensitive mutants of adenovirus single-stranded DNA-binding protein. J. Biol. Chem. **266:**16178–16187.
47. **van der Vliet, P. C., A. J. Levine, M. S. Ensinger, and H. S. Ginsberg.** 1975. Thermolabile DNA binding proteins from cells infected with a temperature-sensitive mutant of adenovirus defective in viral DNA synthesis. J. Virol. **15:**348–354.
48. **van Ormondt, H., and F. Galibert.** 1984. Nucleotide sequences of adenovirus DNAs, p. 73–142. *In* W. Doerfler (ed.), The molecular biology of adenoviruses 2. Springer-Verlag, Berlin.
49. **Voelkerding, K., and D. F. Klessig.** 1986. Identification of two nuclear subclasses of the adenovirus type 5-encoded DNA-binding protein. J. Virol. **60:**353–362.
50. **Wang, Q., X. Jia, and M. H. Finer.** 1995. A packaging cell line for propagation of recombinant adenovirus vectors containing two lethal gene-region deletions. Gene Ther. **2:**775–783.
51. **Yang, Y., F. A. Nunes, K. Berencsi, E. E. Furth, E. Gönczöl, and J. M. Wilson.** 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc. Natl. Acad. Sci. USA **91:**4407–4411.
52. **Yang, Y., F. A. Nunes, K. Berencsi, E. Gönczöl, J. F. Engelhardt, and J. M. Wilson.** 1994. Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. Nat. Genet. **7:**362–369.
53. **Yei, S., N. Mittereder, S. Wert, J. A. Whitsett, R. W. Wilmott, and B. C. Trapnell.** 1994. *In vivo* evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. Hum. Gene Ther. **5:**731–744.
54. **Zsengeller, Z. K., S. E. Wert, W. M. Hull, X. Hu, S. Yei, B. C. Trapnell, and J. A. Whitsett.** 1995. Persistence of replication-deficient adenovirus-mediated gene transfer in lungs of immune-deficient (nu/nu) mice. Hum. Gene Ther. **6:**457–467.